

Effects of cochlear pre-conditioning on cisplatin ototoxicity

Honors Research Thesis

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by  
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**Abstract**

The protective effects of cochlear pre-conditioning have been documented, especially in regard to noise. Conditioning with moderate-intensity noise toughens the ear and makes it more resistant to damage from high-level noise exposures. The goal of the project was to determine whether cochlear pre-conditioning is effective in protecting against the ototoxic effects of cisplatin. Pre-conditioning with noise has shown to reduce hearing loss from cisplatin, but pre-conditioning with cisplatin had never been attempted. 21 Fischer 344 rats, split into control and experimental groups, were used for the test. Saline solution was administered to the control group while cisplatin was given to the experimental group, each increasing doses throughout the conditioning period. Injections were given once every two weeks with a hearing test during off weeks. The threshold of each animal was determined through measuring auditory brainstem responses while the animal was under sedation. After conditioning, each animal was given a large dose of cisplatin (12 mg/kg) and given a hearing test three days later to determine if the pre-conditioning was successful in reducing hearing loss. During the conditioning phase, animals treated with low-dose cisplatin lost weight in the week after the cisplatin exposure, but did not develop any hearing loss. Following the high dose of cisplatin, results showed that the cochlear pre-conditioning with cisplatin did not reduce cisplatin-induced hearing loss. The experimental group had higher thresholds and was in worse health after the final cisplatin dose than the animals conditioned with saline. Since there was no evidence that the saline conditioning affected cisplatin ototoxicity, the conclusion can be made that prolonged exposure to cisplatin, even at lower exposure levels, weakens the animal and exacerbates cisplatin's ototoxicity. Therefore, cochlear pre-conditioning prior to cisplatin exposure cannot be executed using cisplatin itself as the conditioning agent.

## 1. Introduction

Cisplatin (cis-diamminedichloroplatinum (II)) is a widely used, platinum-based chemotherapy drug that has been found to be effective in treating various types of cancer, including those in the testicles, ovaries, lungs, and head and neck. (Cooley et al., 1994). The mechanism responsible for cisplatin's effectiveness against cancer cells is its ability to covalently bind to cell DNA. It then bends and damages the DNA strand, which causes cell destruction through both apoptotic and nonapoptotic methods (Reed & Chabner 2010). However, it has the potential to cause a number of side effects, such as nephrotoxicity (Campbell et al., 1983), myelosuppression (Eagan et al., 1980), and ototoxicity (Hayes et al., 1977), which is of primary interest in this study. The rate of hearing loss varies depending on the patient and the administered dose, and cases have varied between 9% and 90% (Sako, et al., 1987; Hayes et al., 1977). The ototoxic effects of cisplatin begin with the outer hair cells at the base of the cochlea and spread throughout the cochlea as the cisplatin treatment continues (See Figure 1). The outer hair cell damage at the base of the cochlea translates to a high-frequency (6 kHz and above) hearing loss that progresses into the middle frequencies (1.5-6 kHz) as the dose and duration of cisplatin exposure increases (Madasu et al., 1997). Patients with cisplatin-induced hearing loss typically show significantly reduced ability to understand speech, a problem that is more severe in the pediatric population (Knight et al., 2005). Therefore, there is a major need for methods to protect the ear from cisplatin, so that it can be used aggressively to fight cancer, without severely injuring the inner ear.

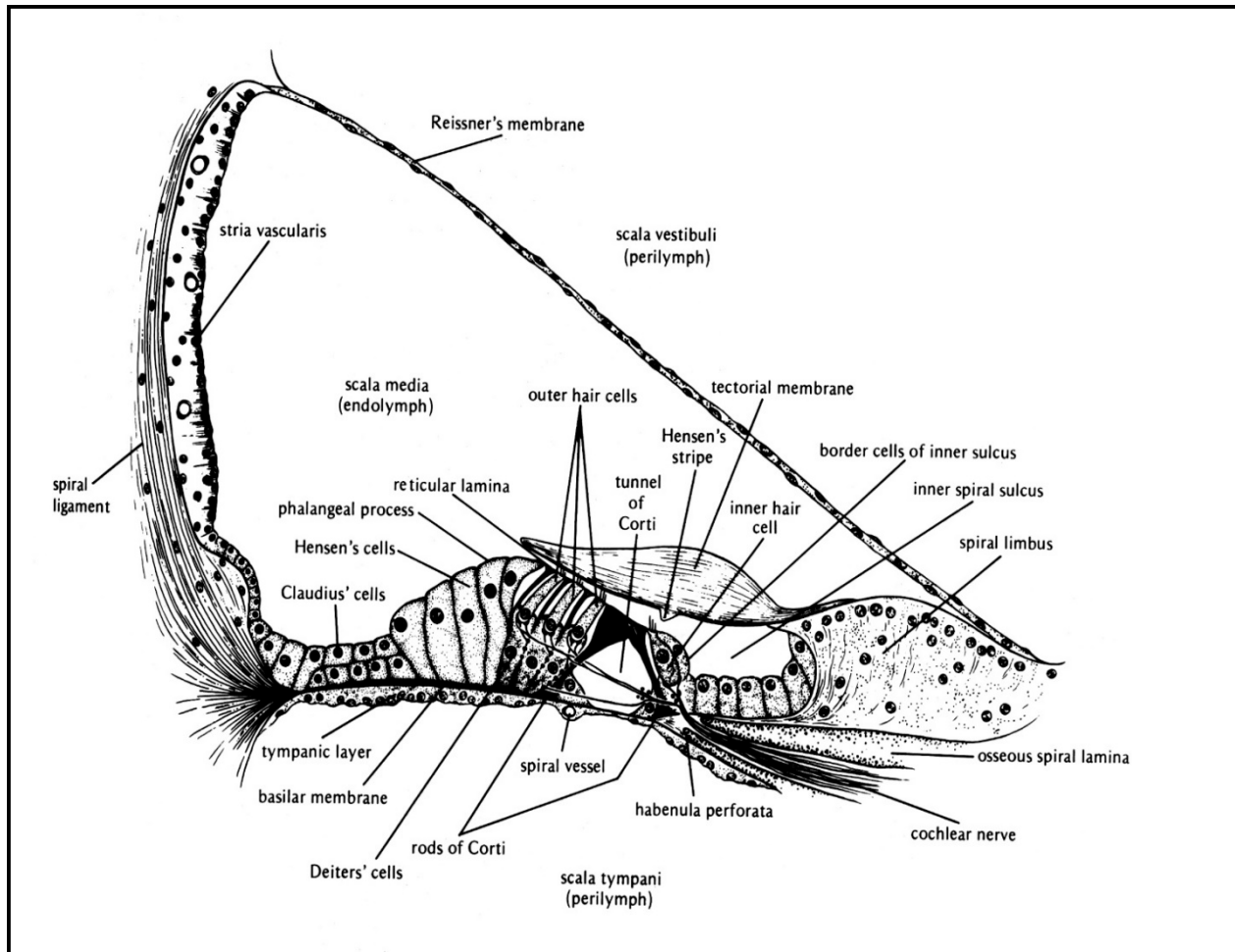


Figure 1. A cross section the center portion of the cochlea, scala media, which shows a detail of the Organ of Corti. (Yost, W. A. (2008). *Fundamentals of Hearing*. (5th ed., p. 86). Bingley, UK: Emerald Publishing Group.)

One of the primary ways in which cisplatin triggers the destruction of outer hair cells is through oxidative stress. This is caused by an imbalance of reactive oxygen species (ROS) and antioxidants. ROS are oxygen-based molecules with an unpaired electron that scavenge electrons from other, more stable molecules, which, when widespread throughout a group of cells, can cause a condition of oxidative stress. Antioxidants are molecules that scavenge ROS and convert them into benign molecules that have reduced potential for toxicity. Oxidative stress occurs in conditions in which the levels of ROS in tissue significantly exceed the levels of antioxidants. Oxidative stress can lead to cell death through DNA damage, plasma membrane injury, and cell death through triggered apoptosis (Halliwell & Gutteridge, 1999). During cisplatin ototoxicity, cisplatin not only increases the ROS levels within the outer hair cells, but depletes the antioxidant levels simultaneously (Yamane et al., 1995; Ravi et al.). The end result is a high level of oxidative stress that can lead to massive cell loss in the cochlea in a short period of time.

Increased ROS levels and the subsequent apoptosis of cochlear hair cells have also been implicated as a potential cause of noise-induced hearing loss, as prolonged exposure to loud noises promotes ROS production and overwhelms available antioxidants (Harris, et al., 2006). Noise pre-conditioning, through gradually increasing noise levels prior to a massive traumatic noise exposure, has been shown to reduce outer hair cell damage (Canlon et al., 1988; Campo et al., 1991; McFadden et al., 1997) as well as altering the pattern of hair cell destruction (Canlon & Fransson, 1994). Furthermore, noise pre-conditioning regimens have also been used in conjunction with ototoxic drugs to produce a protection effect. For instance, noise pre-conditioning has been successfully used to reduce the ototoxic effects of the herbicide paraquat. Cellular exposure to paraquat causes an increase in ROS levels (Harris et. al., 2006). The

underlying mechanism of the protective effects of noise conditioning before either traumatic noise or paraquat exposure is believed to be an increase in antioxidant levels in the hair cells after the pre-conditioning noise (Jacono et al., 1998). The increased antioxidant levels render the cells less susceptible to damage from the traumatic noise. Therefore, it is possible that through cochlear pre-conditioning, the ototoxic effects of cisplatin can be reduced. In another more recent study, where groups of mice were given cisplatin with and without noise pre-conditioning, a significant protective effect resulted in the mice with pre-conditioning. Not only were thresholds significantly reduced, especially in the area of 16 kHz, but outer hair cell death was prevented in the middle cochlear turn (Roy et al., 2013). The purpose of the proposed study is to assess pre-conditioning with low levels of cisplatin, prior to a high-level dose of cisplatin that is known to cause significant cochlear damage and hearing loss. Intra-modal pre-conditioning with cisplatin to prevent cisplatin ototoxicity has never been attempted and could be a useful way to use cisplatin clinically while optimizing its anti-cancer effects and minimizing its ototoxic effects.

### *Expected Results*

The expected result of the experiment was a reduction in the ototoxic effects of cisplatin in the pre-conditioned animals. As cisplatin was introduced into the auditory system at low-dose levels, the hypothesis was that cochlear antioxidant levels would be reduced by the cisplatin doses but the cochlea would compensate by increasing antioxidant production to protect against the increase in ROS levels. As a result, cochlear antioxidant levels would be elevated prior to the high-dose cisplatin exposure, thereby resulting in minimal outer hair cell destruction following the damage phase of the experiment. It was suspected that the control group would

have no antioxidant level increase and, therefore, a higher rate of cell death after the high-dose cisplatin exposure.

## **2. Methods**

### *Subjects*

To test the effects of cochlear pre-conditioning, twenty-one female Fischer 344 rats were used. The animals were acquired from Harlan Laboratories at 2-3 months of age. The rats were kept in a quiet colony and all proceedings involving the animals were approved by The Ohio State University's Institutional Animal Care and Use Committee.

### *Auditory Brainstem Response (ABR) Testing*

In order to perform ABR tests, the animals were anesthetized with a mixture of gaseous isoflurane and oxygen. For initial anesthetization, 4% isoflurane was used with a 1L/min O<sub>2</sub> flow rate. Following anesthesia, the animals were placed in a sound booth with a nose cone supplying a mixture of 1.5% isoflurane and oxygen for the duration of the test. In the sound booth, three platinum electrodes were inserted subcutaneously, one in the vertex, one behind the right pinna, and the ground near the left rear leg of the animal. All stimuli were generated using Tucker Davis Technologies (TDT, Gainesville, FL) SigGen software. Each tone burst was 1 ms in duration, and had a 0.5 ms rise/fall time with no plateau. Stimuli were presented at a rate of 19/sec. Signals were routed to a speaker (TDT Model MF1) positioned at zero degrees azimuth, 6 cm from the vertex of each rat's head. Acoustic stimuli were calibrated prior to each testing session, by recording the output of the speaker with a microphone placed at the animals' head level. The rats' evoked responses were amplified with a gain of 50,000, using a TDT RA4LI headstage connected to an RA4PA pre-amplifier, and bandpass filtered from 100-3000 Hz. Two hundred-fifty sweeps were averaged at each stimulus level using TDT BioSigRz software.

Six frequencies were tested: 5, 10, 15, 20, 30, and 40 kHz, at decreasing 5 dB SPL intervals from 90 dB to 5 dB.

The human ABR consists of the first five waves of electrical activity post-stimulus. These five waves reflect the activity of the structures of the central auditory system as the signal progresses from the cochlea to the primary auditory cortex and they occur within 10 ms of exposure to a noise stimulus (Yost, 2008). In the rat, the ABR is dominated by two positive and two negative peaks. ABRs were recorded and analyzed with BioSigRZ. To determine the threshold of the animals at individual frequencies, the ABRs at each dB SPL were compared. Threshold was defined as the lowest point at which a brainstem response wave could be discerned (See Figure 2).



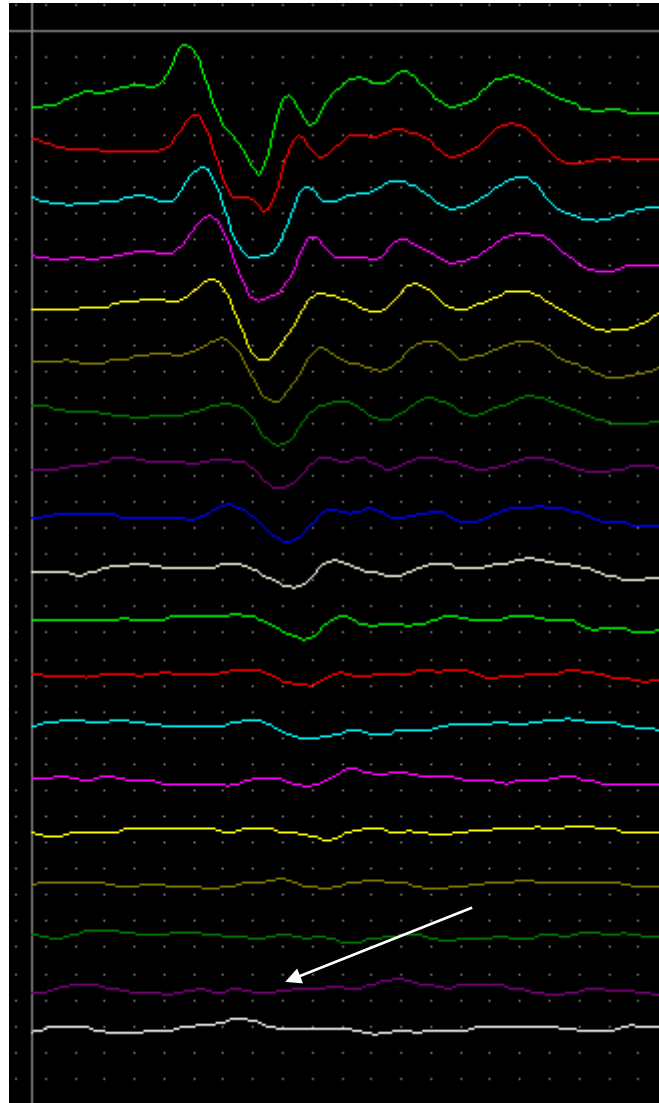


Figure 2. The worksheet view of BioSigRZ showing ABRs from 90 dB to 5 dB. The arrow indicates where threshold would have been measured.

*Pre-conditioning Exposure*

The animals were separated into two groups: one control group and one experimental group. Both groups underwent doses of either saline solution or low-level cisplatin during pre-conditioning phase prior to high-dose cisplatin delivery.

The experiment was divided into three phases: pretest, conditioning, and damage. The pretest phase involved an ABR test to determine baseline thresholds. Following the pretest phase, the animals commenced with the conditioning phase. During the conditioning phase the experimental group received regular, low doses of cisplatin every two weeks. The solution of cisplatin was made by dissolving solid cisplatin into a saline solvent and using a magnetic agitator with mild heat to facilitate the combination. The control group received saline solution instead of cisplatin with the same dosing schedule. All doses during the conditioning phase were administered via intra-peritoneal injection. Week 1 consisted of 2 mg/kg injections of cisplatin or an equivalent volume (0.2-0.4 mL) of saline solution. Week 2 consisted of the second round of ABR tests. Week 3 consisted of cisplatin (3 mg/kg in 0.3-0.6 mL saline) or saline (0.03-0.6 mL) injections and week 4 consisted of the third round of ABR tests. Week 5 was the third dosing week with injections of cisplatin (2 mg/kg) or saline and week 6 was the fourth round of ABR testing. Week 7 was the final week of conditioning injections with cisplatin (3 mg/kg) or saline. Week 8 was the final conditioning week with ABR tests.

Originally, the doses were scheduled to progress from 2 mg/kg to 3 mg/kg for two weeks of dosing and then to 4 mg/kg for the final conditioning week, but due to health concerns levels were reduced for the fifth week. To ensure that any hearing loss was a result of only the damage phase, any animals from the experimental group that exhibited signs of hearing loss would have been removed from the study (this never occurred).

The damage phase consisted of a final, large dose of cisplatin (12 mg/kg) for every animal in both groups and the final post-tests for all animals. Doses were administered via intraperitoneal infusion at a rate of 8mL/hour. Due to the waning health of the animals, the final tests were performed on the third as well as the seventh day of week ten. Furthermore, the animals were regularly hydrated to counteract the effects of renal failure. These measures ensured that as many animals were tested as possible before the necessity of sacrifice.

### **3. Results**

Contrary to the expected result, the experimental group showed a marked increase in threshold following the damage phase. During the Pretest week and the following eight weeks of conditioning, both the control group and the experimental group showed similar thresholds with no significant hearing loss (See Figures 3-8).

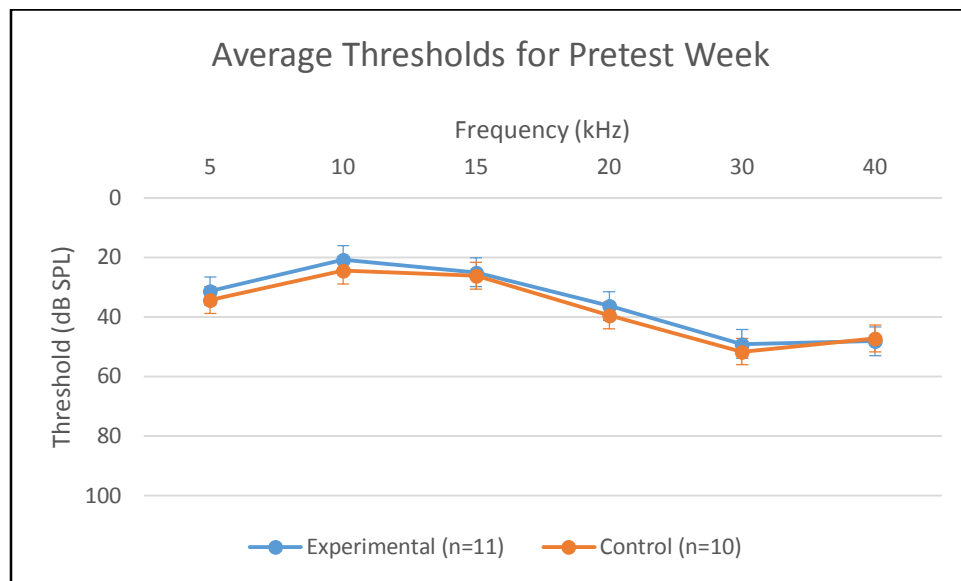


Figure 3. Average thresholds for the pretest week. The Y axis has been inverted to mirror that of an audiogram. Therefore the higher thresholds appear lower on the graph. The experimental group is shown in blue and the control group is shown in orange.

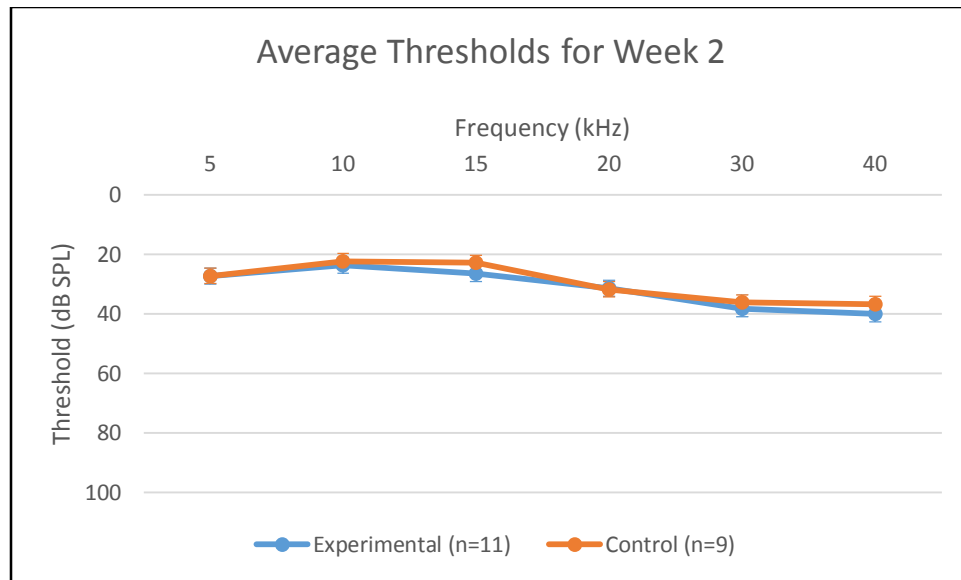


Figure 4. Average thresholds for week 2. The Y axis has been inverted to mirror that of an audiogram. Therefore the higher thresholds appear lower on the graph. The experimental group is shown in blue and the control group is shown in orange.

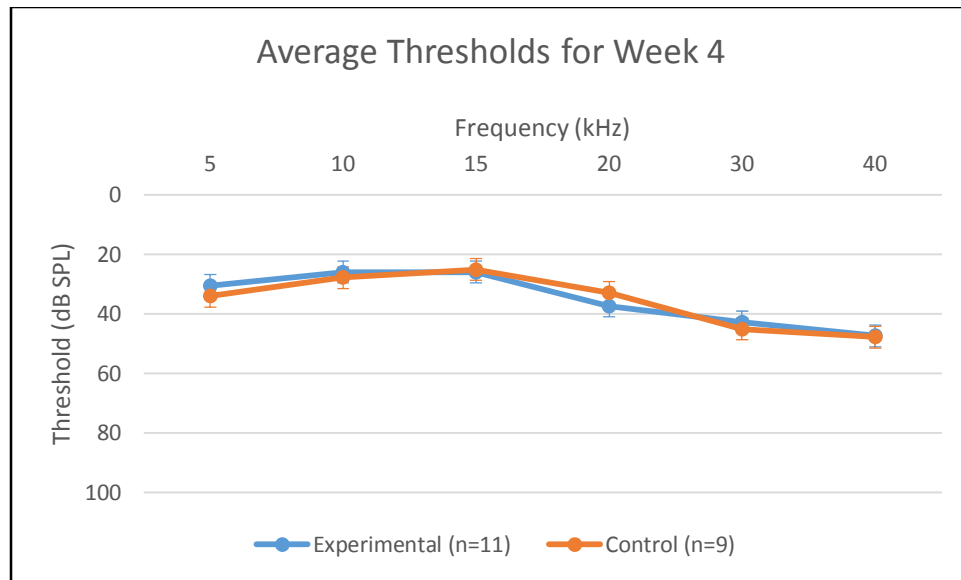


Figure 5. Average thresholds for week 4. The Y axis has been inverted to mirror that of an audiogram. Therefore the higher thresholds appear lower on the graph. The experimental group is shown in blue and the control group is shown in orange.

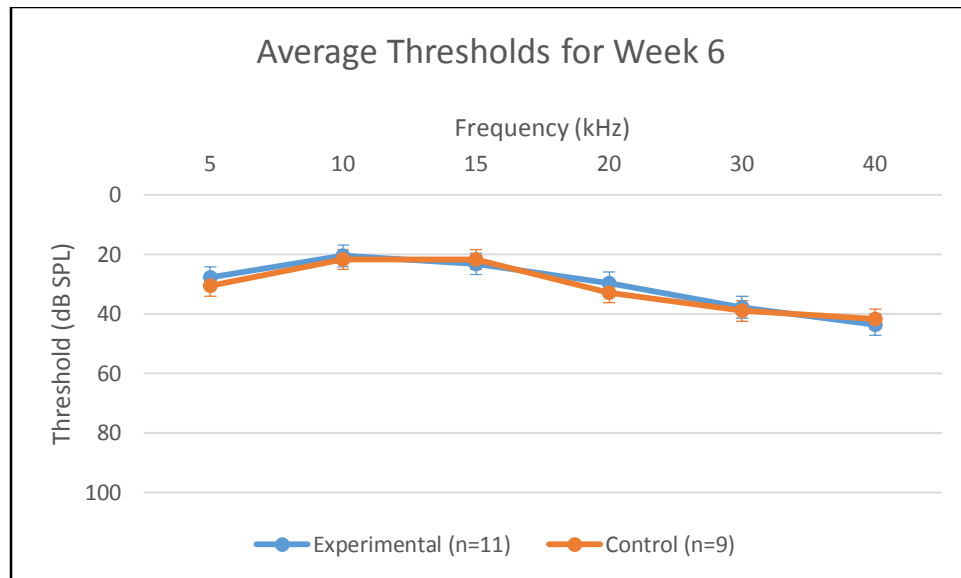


Figure 6. Average thresholds for week 6. The Y axis has been inverted to mirror that of an audiogram. Therefore the higher thresholds appear lower on the graph. The experimental group is shown in blue and the control group is shown in orange.

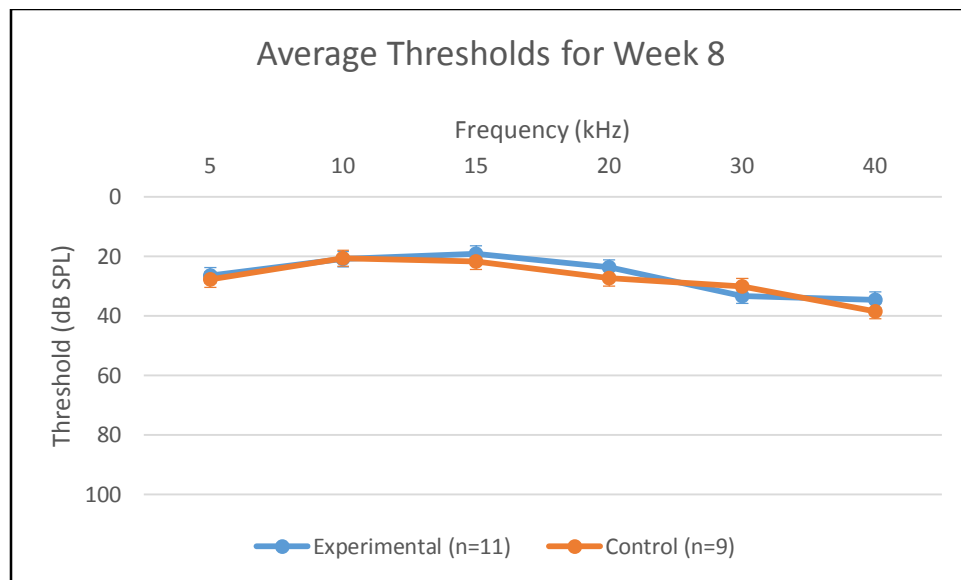


Figure 7. Average thresholds for week 8. The Y axis has been inverted to mirror that of an audiogram. Therefore the higher thresholds appear lower on the graph. The experimental group is shown in blue and the control group is shown in orange.



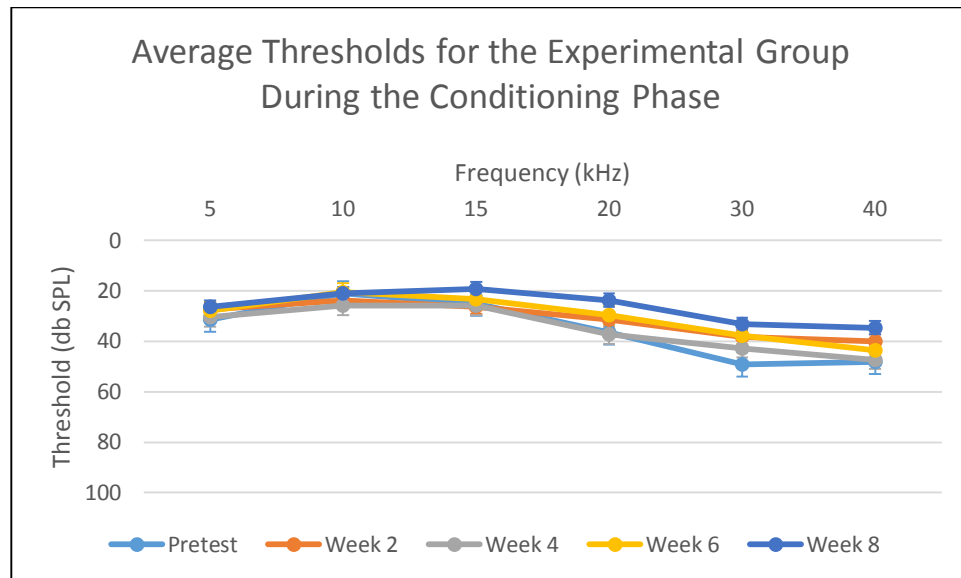


Figure 8. Average thresholds for the experimental group during the conditioning phase. The Y axis has been inverted to mirror that of an audiogram. Therefore the higher thresholds appear lower on the graph. This shows that the experimental group sustained no hearing loss through the first eight weeks of low-level cisplatin conditioning.

On day 3 post-infusion both groups exhibited a sloping hearing loss. The low frequency thresholds of the control group remained normal, while the high frequency thresholds dropped by roughly 30 dB. The experimental group thresholds followed the same shape, but with thresholds that were significantly higher than that of the control group (See Figures 9 and 10).

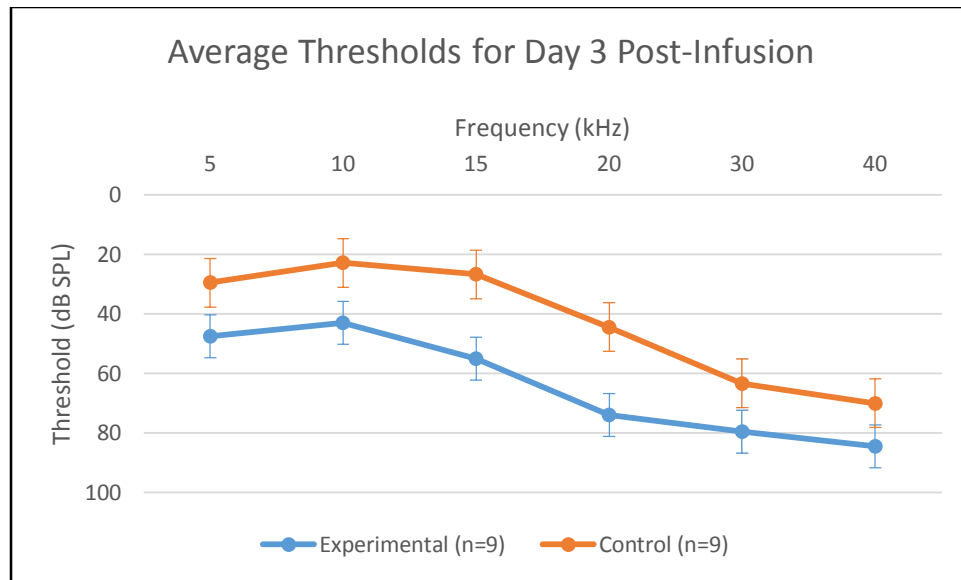


Figure 9. Average thresholds for day 3 post-infusion. The Y axis has been inverted to mirror that of an audiogram. Therefore the higher thresholds appear lower on the graph. The experimental group is shown in blue and the control group is shown in orange.

The significance of the results were determined via repeated measures analyses of variance (ANOVA). A 3-factor repeated measures ANOVA (group x frequency x week) revealed no significant 3-way relationships. There were, however, two 2-way interactions that were significant: frequency x week ( $p < .001$ ) and group x week ( $p < .001$ ). Frequency x week required no additional analysis because it was expected and it was clear that the higher frequencies were more affected by the 12 mg/kg cisplatin dose than the low frequencies. Further 2-factor repeated measures ANOVAs were used to explore the group x week interaction, revealed that only the changes during the final test were statistically significant ( $p < .002$ ). Therefore, it can be concluded that there was a significant threshold change between the experimental group and the control group for day 3 post-infusion tests and day 3 post-tests only.

On day 7 post-infusion the thresholds of the control group in the high frequencies had deteriorated almost to the level of the experimental group, while the low frequency thresholds remained the same. The experimental group showed little change because only three of the animals survived until the end and those animals generally showed the lowest hearing losses at day 3. Day 7 post-infusion tests were not analyzed due to too few animals surviving to the seventh day. By the day 7 post-infusion tests, only six control animals and three experimental animals (out of an original ten control and eleven experimental animals) were alive (See Figures 11 and 12).

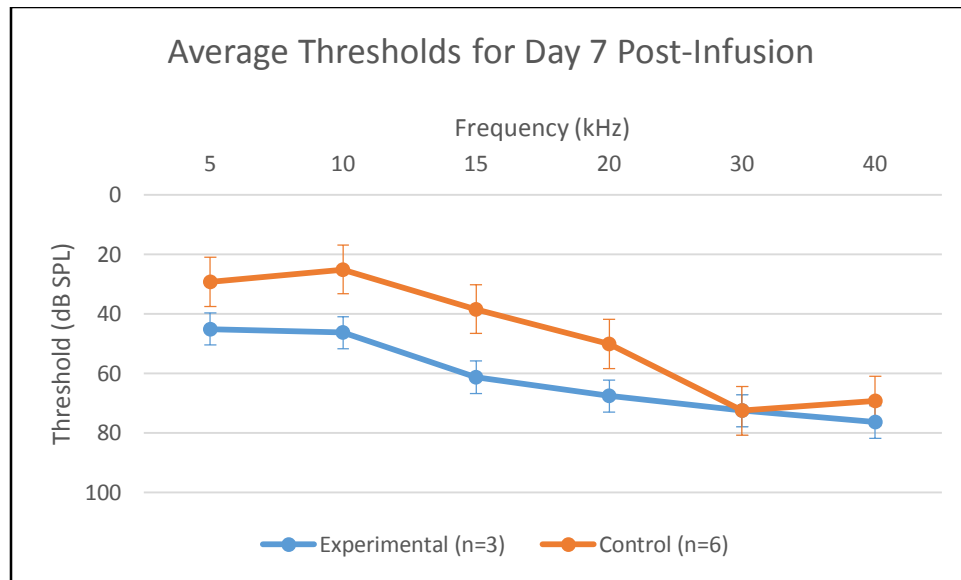


Figure 10. Average thresholds for day 7 post-infusion. The Y axis has been inverted to mirror that of an audiogram. Therefore the higher thresholds appear lower on the graph. The experimental group is shown in blue and the control group is shown in orange.

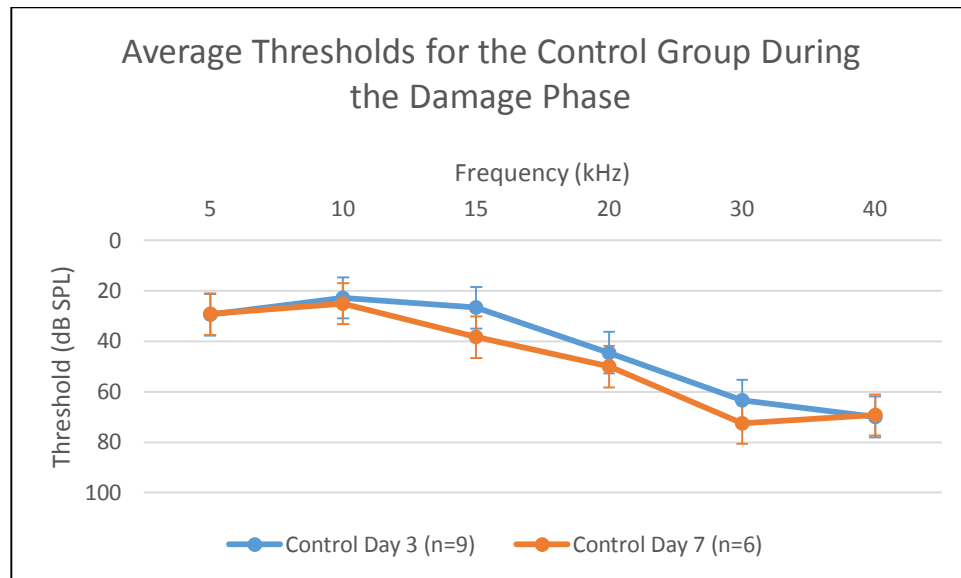


Figure 11. Average thresholds for the control group during the damage phase. The Y axis has been inverted to mirror that of an audiogram. Therefore the higher thresholds appear lower on the graph. Day 3 is shown in blue and day 7 is shown in orange.

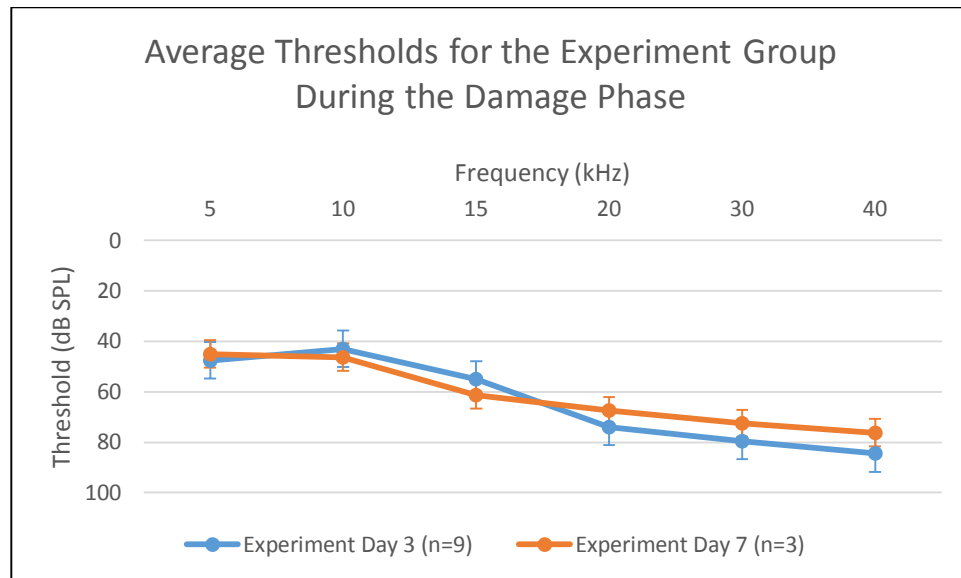


Figure 12. Average thresholds for the experimental group during the damage phase. The Y axis has been inverted to mirror that of an audiogram. Therefore the higher thresholds appear lower on the graph. Day 3 is shown in blue and day 7 is shown in orange.

In addition to the increased hearing loss, the health of the experimental group was worse than that of the control group. The average weight of the experimental group on day 3 post-infusion was 89% of the original weight while that of the control group was 95%. Furthermore, both groups suffered lethargy and effects of nephrotoxicity. These effects were both notably worse in the experimental group than the control and in many cases led to premature death or the necessitation of early sacrifice.

#### **4. Discussion**

The expected outcome was a protective effect from the cisplatin pre-conditioning that would reduce the threshold shift in the experiment group during the damage phase. However, the threshold shift was far greater for the experiment group than that of the control group. This failure to obtain a protective result from the experiment implies that the hair cells did not build up an immunity to the cisplatin as predicted and raised multiple questions as to the reason behind the higher hearing loss in the experimental group.

The first possible cause is that the repeated cisplatin treatments depleted the antioxidant levels in the cells to the point that there was little to no protection from the increased ROS levels during the damage phase. Furthermore, it suggests that if there was an increase in antioxidant levels in the outer hair cells, like that which occurs during noise exposure pre-conditioning, it was nullified by the cisplatin exposure.

The second possible cause is the possibility of permanent susceptibility to hearing loss. While the conditioning phase did not result in significant hearing losses for the experimental group, it is possible that conditioning phase damaged the hair cells and the stria vascularis (See Figure 1) to the point of hearing loss susceptibility. In this scenario, the structures of the inner ear were damaged either very lightly or in a sporadic pattern such that it did not cause a hearing



loss, but were damaged enough to cause a hearing loss with the following case of aural stress. In this case, if the 12 mg/kg cisplatin had been given weeks or months after the conditioning exposures, then the greater hearing loss demonstrated in the experimental group still would have occurred. Bielefeld (2013) demonstrated that a more moderate dose of cisplatin (7 mg/kg) accelerated age-related hearing loss at 20 kHz in Fischer 344/NHsd rats, possibly due to the same smaller or scattered hair cell lesions in the region of the cochlea tuned to 20 kHz.

Neither the experimental nor control group showed significant threshold shift in the high frequencies from day 3 post-infusion to day 7 post-infusion. This can be largely attributed to a ceiling effect, since the animals were only tested to a maximum of 90 dB SPL. By day 3, many animals' thresholds at 30 and 40 kHz were already at 80 dB SPL or higher. Therefore, there was little room for the thresholds to get any higher after day 3. For the experimental group the high frequency thresholds were reduced on the seventh day (See Figure 11). This is likely because the most severely hearing-impaired rats were also the sickest rats, and they died or were sacrificed before the testing on the seventh day, leaving only those least affected to be tested on day 7.

In retrospect, cisplatin's tendency to deplete antioxidants should have been taken into consideration. Perhaps a regimen of antioxidants should have been provided during the conditioning phase to rule out the possibility of antioxidant depletion and, possibly, permanent susceptibility. Furthermore, the negative results could possibly have been predicted due to the increased susceptibility of the cochlea to damage while being exposed to cisplatin. For instance, chinchillas receiving both cisplatin and exposure to high noise levels showed increased hearing loss and cochlear damage compared to those only receiving cisplatin (Gratton et al., 1990).

In terms of future research, a follow up protection study is being performed to determine whether or not noise pre-conditioning and the subsequent increase of antioxidant levels will protect against cisplatin exposure. Since the pre-conditioning agent in the subsequent study will not deplete antioxidants while increasing ROS levels, there is optimism about the results.

Finally, to determine the likelihood of a permanent susceptibility situation, a study could be undertaken in which low doses of cisplatin are administered without an immediate damage phase. This could bring the inner ear structures to the point of permanent susceptibility, but would not immediately push them over the threshold. Sometime later, a damage phase of either cisplatin or noise would be administered.

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